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A COLORIMETRIC ESTERASE ASSAY

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Using rat serum as a source of esterase activity, we describe a quick and efficient method of taking advantage of the water solubility of the paranitrophenoxide anion. Esterase activity is terminated by adding chloroform and releasing paranitrophenol converted to the 400 nm absorbing paranitrophenoxide anion by adding 0.2 M phosphate buffer, pH 9.0. Paranitrophenoxide is quantitatively partitioned into the aqueous phase, while leaving unhydrolyzed substrate in the organic (chloroform) phase. Partitioned paranitrophenoxide is monitored spectrophotometrically at 400 nm. This method allows quantitation either by direct comparison of identically treated standards or by utilization of appropriate Molar Extinction Coefficients independent of assay pH.

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PREFACE

The work described in this report was authorized under Contract No. DAAA15-89-C-0008. This work was started in March 1989 and completed in August 1989. The experimental data are recorded in laboratory notebooks in the possession of the contractor.

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Animal Resources, National Research Council.

This report has been approved for release to the public.

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A COLORIMETRIC ESTERASE ASSAY

1. INTRODUCTION

Esters of paranitrophenol are widely used chromogenic substrates due to their being relatively stable and having great sensitivity in detecting esterase activity.[1,2] However, because paranitrophenol has a pKa value of 7.2 (dissolved in water at 25°C, cf. ref. 3), the protonated form predominates at neutrality. A potential problem that arises in quantitating degree of hydrolysis of these esters is that absorbance maxima for the yellow chromophoric product, paranitrophenoxide, and its protonated colorless form, paranitrophenol, are significantly different.[1] Any attempt to convert to the paranitrophenoxide anion by reducing hydrogen ion concentration without first removing unhydrolyzed substrate may result in either significant enzymatic hydrolysis if the pH optimum of the esterase of interest is broad and/or nonenzymatic, alkaline hydrolysis of unhydrolyzed substrate. In either case, the accuracy and sensitivity of the assay are compromised. In this report, we describe a quick and efficient method of extraction, taking advantage of the water solubility of the chromophoric hydrolysis product of paranitrophenyl-esters, paranitrophenoxide anion.

2. MATERIALS AND METHODS

Paranitrophenylacetate, paranitrophenylbutyrate, paranitrophenol, ^{14}C-paranitrophenol, sodium chloride, and sodium phosphate were obtained from Sigma Chemical Company, St. Louis, MO. Chloroform was obtained from Fisher Chemical Company, Houston, TX. Male, Sprague-Dawley rats were purchased from Charles River Laboratories, Wilmington, MA. Liquiscint scintillation fluid was purchased from National Diagnostics, Manville, NJ.

2.1 Preparation of Serum.

Rats were decapitated and trunk blood collected. Blood was allowed to clot on ice for 30 minutes and serum carefully removed by aspiration.

2.2 Esterase Assay.

Assays contained in a final volume of 100 µl, 20 µl 0.1 M phosphate buffer, pH 7.0, 45 µl distilled water, 10 µl substrate (25 mM) dissolved in acetone and 10 µl of serum. Reaction mixtures were kept in an ice slurry during addition of components. Prior to reaction initiation by addition of substrate, reaction mixtures were incubated at 35°C for 30 seconds. Reactions were terminated after 1 minute by addition of 1.0 mL cold chloroform and shaken vigorously. Unless indicated otherwise, 1.5 mL 0.2 M phosphate buffer, pH 9.0, was added following extraction to facilitate partitioning of paranitrophenoxide anions. Reaction mixtures were vigorously shakened and centrifuged at 2000 rpm for 5 minutes at 5°C. A 1.0 mL aliquot of the upper, aqueous phase was removed and partitioned paranitrophenoxide anion was quantitated spectrophotometrically at 400 nm.

Paranitrophenol served as standard and was treated in identical fashion as the experimental samples. Blanks consisted of incubation mixtures to which serum was added after termination of the reaction with chloroform. Each experimental and corresponding blank was carried out in triplicate.

3. RESULTS

Absorbance values obtained for different ratios of conjugate-base (paranitrophenoxide) and acid (paranitrophenol) are shown in the Figure. As indicated in the Figure, significant differences in absorbance at 400 nm occur between pH values 6.2 and 8.2 (1 log unit above and below the pKa value). Shown in the same figure (open circles) is the effect of decreasing hydrogen ion concentration (pH 6.2-10.2) upon nonspecific hydrolysis of paranitrophenyl-acetate. An identical curve was obtained for paranitrophenylbutyrate (data not shown).

Using carbon-14 labelled paranitrophenol, the efficiency of extraction was evaluated at two different ion concentrations. As indicated in Table 1, paranitrophenol and paranitrophenoxide were extracted quantitatively (greater than 95%) into the organic and aqueous phases, respectively, depending upon partitioning buffer pH. Shown in Table 2 is a comparison of serum esterase activity carried out at neutrality (pH 7.0) but partitioned at various hydrogen ion concentrations. Absorbance values were observed to increase with increasing conjugate acid-base ratios after termination of the reaction. As indicated in the Table, enzyme activity (nmoles paranitrophenol cleaved/min/mL serum) is significantly underestimated when the protonated species predominate.

4. DISCUSSION

Paranitrophenol is the yellow chromophoric product of the hydrolysis of paranitrophenylesters.[4] However, the yellow paranitrophenoxide anion is the absorptive species at 400 nm. As shown in the Figure, increasing the ratio of conjugate base (paranitrophenoxide) to acid (paranitrophenol) increases significantly absorbance at 400 nm. When the paranitrophenoxide anion/ paranitrophenol ratio is 1.0, the midpoint of the curve should correspond approximately to an optical density of 0.5. We observed an optical density of approximately 0.35. In our calculations, we have used the pKa value of 7.2,[3] which was determined using water as the solvent. These values may differ slightly depending upon the solvent used. However, it is apparent that conversion of released paranitrophenol to paranitrophenoxide gives rise to a much more sensitive assay. Partitioning at pH 9.0 not only favors formation of conjugate base (paranitrophenoxide) greater than 60 to 1 but equally important, results in less than 5% nonenzymatic hydrolysis. Extraction of paranitrophenol and paranitrophenoxide into the organic and aqueous phase, respectively, is quantitative as evidenced by greater than 95% recovery of carbon-14 labelled paranitrophenol under acid (pH 5.0) and alkaline (pH 9.0) partitioning conditions (Table 1).

Some investigators choose to use the Molar Extinction Coefficient to quantitate released paranitrophenol.[5-7] However, most physiologic assays are conducted between pH 6.5-7.5 and unless carried out under identical conditions,

used to derive Molar Extinction Coefficients, considerable error arises in the determination of extent of hydrolysis. In this pH range, a significant quantity of released paranitrophenol is protonated, resulting in reduced sensitivity at 400 nm (Table 2).

One way to alleviate this problem is to add alkaline buffer to the reaction mixture, thus converting released paranitrophenol to the paranitrophenoxide anion. However, above pH 9.0, nonenzymatic, alkaline hydrolysis of paranitrophenylesters increases substantially. As shown in the Figure, maximum absorbance is achieved by adding pH 9.0 buffer as evidenced by no increase in absorbance following addition of buffer containing lower concentration of protons (pH 10.2). Thus, as long as the buffering capacity at pH 9.0 is significant, there is no need to further decrease the proton concentration (e.g., 10).

Quenching the reaction with chloroform achieves two goals: 1) esterase activity ceases to occur and 2) unhydrolyzed substrate is extracted into the organic phase while released paranitrophenol is converted to the water soluble, paranitrophenoxide anion following aqueous, alkaline partitioning. The method described in this report allows quantitation of paranitrophenoxide either by direct comparison of identically treated paranitrophenol standard or by utilization of appropriate Molar Extinction Coefficients independent of assay pH.

Assay mixtures contained 0.2 M buffer at indicated hydrogen ion concentrations and 25 nmoles of paranitrophenol or paranitrophenyl acetate in a final volume of 100 µl. Partitioning following chloroform extraction was achieved by adding 1.5 mL of 0.2 M buffers (pH 5.2, 6.2, 7.2, 8.2, 9.2 and 10.2). One milliliter aliquots were removed and brought to a final volume of 1.0 mL by addition of 500 µl of the respective buffers. Blanks consisted of assay mixtures containing no paranitrophenol or paranitrophenylacetate.

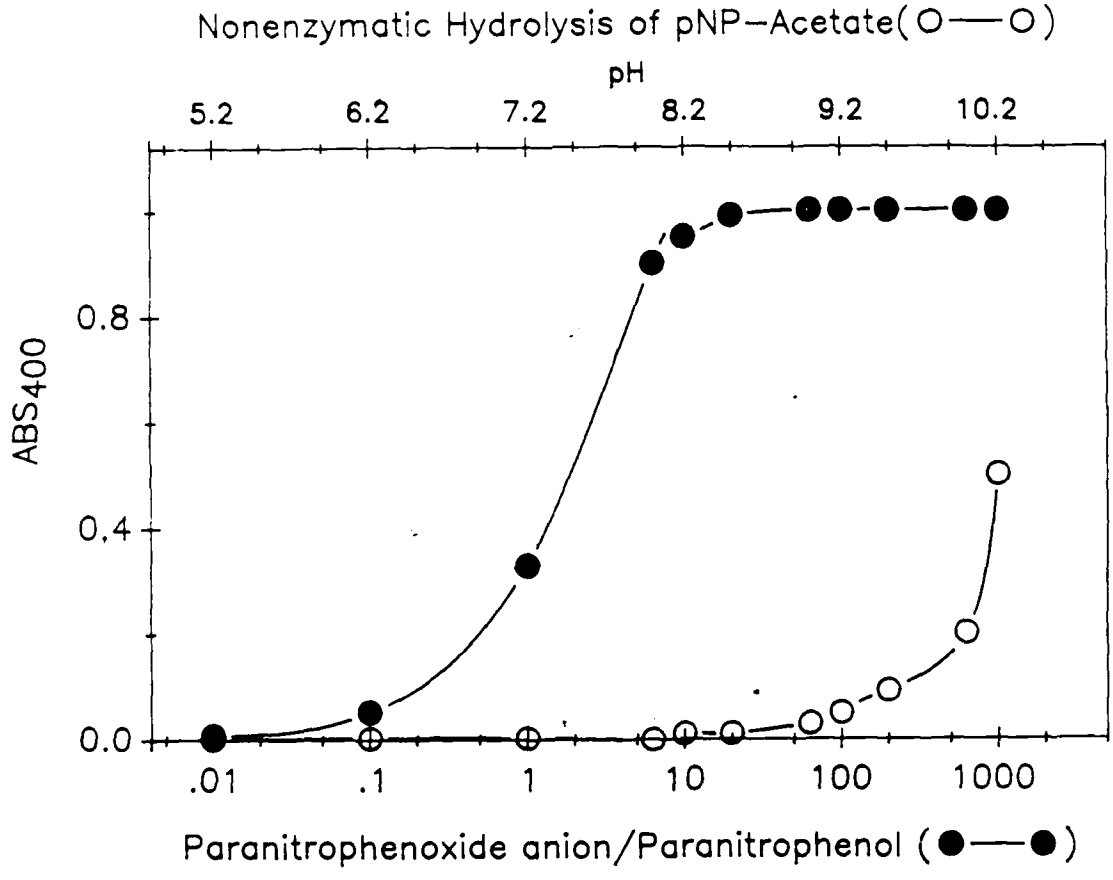

Figure. A Comparison of Various Conjugate Acid-Base Ratios on Absorbance at 400 nm

Table 1. Efficiency of Chloroform-Buffer Partitioning

	Organic	Aqueous
pH 5.0	960,650 (96)	23,300 (2.3)
pH 9.0	22,300 (2.2)	962,330 (96)

Legend: Reaction mixtures containing 1.0×10^6 cpm carbon-14 labelled paranitrophenol were chloroform extracted and partitioned with pH 5.0 and 9.0 phosphate buffer as previously described. Aliquots (100 µl) of both organic and aqueous phases were removed and radioactively determined. Values shown in parentheses represent the percentage of radioactive paranitrophenol recovered in the organic and aqueous phase of each assay mixture.

Table 2. Comparison of Absorbance, Conjugate Acid-Base Ratios and Serum Esterase Activity Obtained by Partitioning at Various Hydrogen Ion Concentrations

Assay	A_{400}	A^-/HA	Sp. Activity
1	0.047	0.63/1	1.4
2	0.149	6.3/1	4.5
3	0.198	63.0/1	6.0
4	0.198	630/1	6.0

Legend: Enzyme assays were carried out at pH 7.0 and quenched by addition of chloroform. Partitioning was accomplished by addition of 0.2 M buffer adjusted to pH 7.0. (Assay 1), pH 8.0 (Assay 2), pH 9.0 (Assay 3), and pH 10 (Assay 4). Specific activity (nmoles paranitrophenol released/min/mL serum) was obtained by direct comparison to a standard paranitrophenol curve, chloroform extracted and partitioned with 0.2 M phosphate buffer, pH 9.0.

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